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Phenotypic and molecular typing of *Listeria monocytogenes* isolated from the processing environment and products of a sandwich-producing plant

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Phenotypic and molecular typing of *Listeria monocytogenes* isolated from the processing environment and products of a sandwich-producing plant

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1. Abstract

Listeria monocytogenes as a food-borne pathogen has significant public health and economic impacts. The present study investigated the diversity of *L. monocytogenes* in a Swiss sandwich-producing plant over a 12-month period. *L. monocytogenes* were detected by culture after enrichment in 70 (3.5%) of 2'028 environmental swabs and 16 (7.4%) of 217 samples from ingredients and sandwiches. Of the 86 *L. monocytogenes* strains, 93% belonged to serotype 1/2a and genetic lineage II. Rep PCR and PFGE analysis yielded each six profiles. Sixty-seven (77.9%) strains belonged to only one genotype, which was repeatedly found on/in slicers, conveyor belts, tables, a bread-feeding machine, spattles, air blow-guns, salmon, and egg sandwiches. Strains of this genotype persisted for more than nine months in the processing environment, in particular on slicers and conveyor belts, which probably contributed to the contamination of sandwiches. After revision of the cleaning and disinfection procedures, *L. monocytogenes* were no longer found on slicers, conveyor belts, or in products. Besides, these results emphasize the importance of environmental monitoring schemes to identify potential contamination sources and as an early warning system for food business operators.

Keywords:
Listeria monocytogenes; Genetic diversity; Persistence; Ready-to-eat food-processing plant; Sandwiches

2. Introduction

Listeria (L.) monocytogenes as a food-borne pathogen has significant public health and economic impacts. Human infections primarily result from eating contaminated food and may lead to serious and potentially life-threatening listeriosis. Pregnant women, neonates, elderly, or immunocompromised people are particularly susceptible to listeriosis, which typically presents as septicemia, meningitis, or meningoencephalitis (Doganay, 2003; Vázquez-Boland et al., 2001). Because of its high case fatality rate, listeriosis ranks among the most frequent causes of death due to food-borne illness (CDC, 2009; Mead et al., 1999; Swaminathan & Gerner-Smidt, 2007). Reports show that *L. monocytogenes* infections are responsible for high hospitalization rates and have been linked to sporadic episodes and large outbreaks (Jemmi & Stephan, 2006; Varma et al., 2007; Warriner & Namvar, 2009). In the European Union (EU), a total of 1'381 confirmed human cases of listeriosis (0.3/100'000) have been reported in 2008 (EFSA, 2010). In several states of the EU, the annual incidence rate has thereby increased over the last few years, especially in the elderly population.

Furthermore, *L. monocytogenes* is of great economic relevance for food business operators because contamination of food products might result in large recalls and changed buying behavior of consumers (CDC, 2002; Jemmi & Stephan, 2006; Wong, Street, Delgado, & Klontz, 2000). Amongst other foods, ready-to-eat products have recently often been implicated in recalls and outbreaks (CDC, 2002; Dawson, Evans, Willby, Bardwell, Chamberlain, & Lewis, 2006; Swaminathan & Gerner-Smidt, 2007). Thereby, the ability of *L. monocytogenes* to persist in food-processing environments and to grow at low environmental temperatures present major challenges for the food industry and food safety (Tasara & Stephan, 2006; Warriner & Namvar, 2009).

According to the EU food safety legislation the onus of compliance is placed on food business operators, which must apply compulsory self-checking programs following the hazard analysis critical control point (HACCP) approach and must ensure that their food products meet the regulatory requirements. Thus, Regulation (EC) No. 2073/2005 sets out microbiological criteria for food-borne pathogens on foodstuffs (Anonymous, 2005). Moreover, this regulation requires manufacturers of ready-to-eat foods to examine the processing environment for *L. monocytogenes* as part of their sampling schemes.

Such examinations along with data from food products form the basis to monitor the *L. monocytogenes* situation in a food-processing plant. In the present study, we investigated the occurrence and genetic diversity of *Listeria monocytogenes* in a Swiss sandwich-producing plant over a 12-month period. The aim was to evaluate the potential persistence of *L. monocytogenes* in the processing environment and to identify possible contamination sources and routes for food products.

3. Materials and Methods

3.1. Food-processing plant and sampling

This study was based on investigations carried out within 12 months (November 2008 to November 2009) in a ready-to-eat food-processing plant. In the processing room of this plant, five sandwich production lines were located. Weekly, 48'000 sandwiches were produced. Packaged sandwiches were distributed to retail shops throughout Switzerland.

To evaluate the occurrence of *Listeria* and especially *L. monocytogenes*, 1'192 samples from the equipment of the sandwich processing lines (e.g. slicers, knives, or conveyor belts), 307 samples from the environment (e.g. drains, walls, or floors), and 217 samples from ready-to-eat ingredients pre-handled in the plant (e.g. salmon, ham, or salami sliced in the examined plant) and sandwiches (e.g. tuna, salmon, meat, or egg sandwiches) were collected during the working process. Additionally, 529 samples from the equipment and environment were obtained after cleaning and disinfection. Sampling comprised two phases. The distribution of samples in the first sampling phase (November 2008 to July 2009) is shown in Table 1. In the second sampling phase (October to November 2009), 249 samples from the equipment (n=207) and environment (n=42) collected after cleaning and disinfection, and 29 samples from ingredients and sandwiches (final products) were investigated. The aim of the first phase was to evaluate the basic situation and the potential persistence of *L. monocytogenes* strains in this plant, whereas the second phase evaluated the effect of revised cleaning and disinfection procedures and focused on identified problem areas. Sampling was performed twice a week and comprised about 80 samples per visit.

Samples from the equipment and environment were collected with cotton swabs. From ingredients and final products, 25 g were collected in each case. Samples were subsequently placed into cool boxes and microbiological examinations were carried out within 4 h after sampling.

3.2. *Listeria* detection and identification

Examinations for *Listeria* were performed using a two-step enrichment procedure. Each swab was incubated for 24 h at 30 °C in 20 ml of Half-Fraser broth (Oxoid Ltd., Hampshire, United Kingdom). Ingredient and final product samples were incubated for 24 h at 30 °C in 225 ml of Half-Fraser broth (Oxoid Ltd.). A subset of 0.1 ml was then incubated in 10 ml of Fraser broth (Oxoid Ltd.) for 24 h at 37 °C. Subsequently, subsets were plated onto Palcam agar (Merck Eurolab GmbH, Darmstadt, Germany), which were then incubated for 48 h at 37 °C. *Listeria*-like colonies were streaked onto Oxoid Chromogenic Listeria Agar (Oxoid Ltd.) supplemented with Listeria Selective Supplement and Listeria Differential Supplement (Oxoid Ltd.) and incubated for 48 h at 37 °C. Presumptive *L. monocytogenes* colonies on the chromogen agar were streaked onto sheep blood agar (Oxoid Ltd.) for appraisal of hemolysis. To identify other *Listeria* species, the API Listeria Identification Kit was used (bioMérieux SA, Geneva, Switzerland) in accordance with the manufacturer's instructions.

3.3. Characterization of *L. monocytogenes* strains

Isolated *L. monocytogenes* strains were characterized by serotyping, determination of genetic lineages, repetitive element PCR (Rep PCR) typing, and pulsed-field gel electrophoresis (PFGE) analysis. Serotyping was performed at the Swiss National Center for Listeriosis (Lausanne, Switzerland). For the determination of genetic lineages, the ASO-PCR multiplex system targeting the *prf*A virulence gene cluster was applied (Ward, Gorski, Borucki, Mandrell, Hutchins, & Pupedis, 2004). Based on phylogenetic analysis, *L. monocytogenes* is separated into three genetic lineages (Liu et al., 2006). Rep PCR was performed as described by Jeršek et al. (1999).

Thereby, the repetitive extragenic palindrome was amplified using forward primer REP1R-I (5-IIIICGICGICATCIGGC-3) and reverse primer REP2-I1 (5-ICGICTTATCIGGCCTAC-3). Furthermore, *L. monocytogenes* were characterized by PFGE following the CDC PulseNet protocol (http://www.cdc.gov/pulsenet/protocols.htm) and modifications in accordance with Benjamin and Datta (1995). Briefly, colonies from blood agar were resuspended in cell suspension buffer (OD_{610}=1.3). For plug preparation, 300 µl of cell suspensions were mixed with 300 µl of 1.2% chromosomal grade agarose (Bio-Rad Laboratories, Basel, Switzerland) and cells were lysed by lysozyme and proteinase K treatment. After washing with Tris-EDTA buffer, DNA agarose plugs were incubated over night in the presence of *Apa*I (Promega, Madison, Wis., USA) following the manufacturer's instructions. Macrorestriction fragments were separated in 1% pulsed field certified agarose gel at 6 V/cm in 10 X Tris-Borate-EDTA buffer cooled to 14 °C in a CHEF-DR III system (Bio-Rad Laboratories). The pulse times were ramped from 4 to 40 s for 19 h and an angle of 120°. Gels were stained with ethidium bromide and visualized under UV light transillumination with Gel Doc (Bio-Rad Laboratories).

4. Results

4.1. Prevalence and distribution of *Listeria*

Of the 1'779 equipment and environmental samples collected in the first sampling phase (November 2008 to July 2009), 65 (3.7%) were positive for *Listeria* spp. (Table 1). The 60 *Listeria* isolates collected during the working process were identified as *L. monocytogenes* (59 strains) and *Listeria welshimeri* (one strain), whereas the five *Listeria* isolates collected after cleaning and disinfection were identified as *L. monocytogenes*. The origin of the 64 *L. monocytogenes* strains is shown in Table 2. Thereby, 28 strains (43.8%) originated from three slicers, which tested repeatedly positive. Besides, in the second sampling phase (October to November 2009), six (2.4%) of the 249 samples collected from the equipment and environment after cleaning and disinfection were positive for *Listeria* spp. Positive samples were thereby only detected during the first two visits.

All six isolates were identified as *L. monocytogenes* and originated from a bread-feeding machine repeatedly testing positive, a water hose, and a drain (Table 2).

Furthermore, 22 (11.7%) of the 188 samples from pre-handled ingredients and final products collected in the first sampling phase tested positive for *Listeria* spp. (Table 1), whereas *Listeria* spp. were not detected among the 29 samples collected in the second sampling phase. Of the 22 *Listeria* isolates, 16, 4, 1, and 1 were identified as *L. monocytogenes*, *L. welshimeri, L. seeligeri,* and *L. innocua*, respectively. The 16 *L. monocytogenes* strains originated from sliced salmon, sliced ham, tuna sandwiches, egg sandwiches, and meat sandwiches (Table 3), whereas *L. welshimeri*, *L. seeligeri*, and *L. innocua* were only found in sliced salmon.

4.2. Characterization of *L. monocytogenes* strains

Of the 64 *L. monocytogenes* strains obtained from the equipment and environment in the first sampling phase (during the working process and after cleaning and disinfection), 61 belonged to serotype 1/2a and genetic lineage II, whereas the remaining three strains belonged to serotype 1/2b and genetic lineage I (Table 2). The six *L. monocytogenes* strains obtained from the equipment and environment in the second sampling phase (after cleaning and disinfection) belonged to serotype 1/2a and genetic lineage II. Of the 16 *L. monocytogenes* strains obtained from ingredients and final products, 13 belonged to serotype 1/2a and genetic lineage II, and three belonged to serotype 1/2b and genetic lineage I (Table 3).

Genotyping of all the 86 isolated *L. monocytogenes* strains yielded six different patterns using Rep PCR typing (a to f) and six different patterns using PFGE analysis (A to F). The 64 *L. monocytogenes* strains obtained from the equipment and environment in the first sampling phase comprised three different genotypes (Table 2), and the great majority (84.4%) of them belonged to only one genotype (a, A). All 48 strains obtained from the equipment of the processing lines belonged to the predominant genotype (a, A). Strains of this genotype were thereby repeatedly found on slicers, conveyor belts, tables, and spattles. The 16 strains obtained from the environment comprised three different genotypes (a, A; b, B; c, C). Thereby, strains from water hoses, air blow-guns, and floors repeatedly belonged to a certain of these genotypes.

Besides, the six *L. monocytogenes* strains obtained from the equipment and environment in the second sampling phase (after cleaning and disinfection) belonged to two different genotypes (a, A; c, C) (Table 2). Thereby, the four strains from the bread-feeding machine belonged to the predominant genotype (a, A). Furthermore, the 16 *L. monocytogenes* strains obtained from ingredients and final products comprised five different genotypes (Table 3). More than half of these 16 strains belonged to only one genotype (a, A).

5. Discussion

In the examined sandwich-producing plant, *L. monocytogenes* were detected within 12 months in 3.5% of the samples obtained from the equipment and environment during the working process and after cleaning and disinfection. Besides, *L. monocytogenes* were also found in ingredients pre-handled in the examined plant and in final products. Salmon sliced in the plant, and sandwiches (tuna, egg, and meat) thereby tested repeatedly positive. In previous studies, fish and seafood have been identified as foods frequently contaminated with *L. monocytogenes* (Jemmi, Pak, & Salman, 2002; Rørvik, Aase, Alvestad, & Caugant, 2000). Similarly, *L. monocytogenes* are found in sandwiches, which have also been associated with outbreaks in the United Kingdom (Dawson et al., 2006; Little, Barrett, Grant, & McLauchlin, 2008; Little, Sagoo, Gillespie, Grant, & McLauchlin, 2009; Pesavento, Ducci, Nieri, Comodo & Lo Nostro, 2010; Wilson, 1996). However, data, especially characterization data of *L. monocytogenes* from sandwich-producing plants were so far scarce in literature.

The great majority (93%) of the 86 *L. monocytogenes* strains isolated in the present study from the processing environment, ingredients, and final products belonged to serotype 1/2a and genetic lineage II. Based on phylogenetic analysis, genetic lineage II covers serovars 1/2a, 1/2c, 3a, and 3c (Liu et al., 2006). Strains of serotype 1/2a are frequently found in food products (Little et al., 2009; Swaminathan & Garner-Smidt, 2007; Varma et al., 2007).

The majority of human cases are associated with *L. monocytogenes* of serotypes 1/2a, 1/2b, and 4b and the proportion associated with strains of serotype 1/2a has increased in recent years (Allerberger & Wagner, 2010; Lukinmaa, Miettinen, Nakari, Korkeala, & Siitonen, 2003; Parihar et al., 2008).

Genotyping of *L. monocytogenes* of this strain collection by Rep PCR and PFGE analysis resulted in a comparable discriminatory power and each method yielded six different patterns (Rep PCR profiles a to f, PFGE profiles A to F). Both methods have been successfully applied in previous studies for typing of *L. monocytogenes* (Brosch, Brett, Catimel, Luchansky, Ojeniyi, & Rocourt, 1996; Chou & Wang, 2006; Jeršek et al., 1999). In the present study, 82.9% of the 70 strains isolated from the processing environment and more than half of the 16 strains isolated from ingredients and final products belonged to only one genotype (Rep PCR profile a, PFGE profile A). Strains of this genotype were repeatedly found in samples from slicers, conveyor belts, tables, a bread-feeding machine, spattles, air blow-guns, salmon sliced in the plant, and egg sandwiches. The second most common genotype (Rep PCR profile c, PFGE profile C) comprised nine strains from the processing environment and three strains from final products. Strains of this genotype were repeatedly found in samples from floors and tuna sandwiches. Thereby, *L. monocytogenes* of these two predominant genotypes (a, A; c, C) were obtained from the processing environment in both sampling phases and both during the working activity and after cleaning and disinfection. Interestingly, half of the six *L. monocytogenes* strains from water hoses belonged to a genotype not detected in other samples, and these were the only environmental strains belonging to serotype 1/2b and genetic lineage I.

Overall, *L. monocytogenes* of two predominant genotypes persisted for more than nine months in the processing environment of the examined plant ("in-house-flora"). This emphasizes the ability of certain *L. monocytogenes* strains to become established in the processing environment (Martinez et al., 2003; Norton, McCamey, Gall, Scarlett, Boor, & Wiedmann, 2001; Wulff, Gram, Ahrens, & Fonnesbech-Vogel, 2006). Persistence thereby might be related to differences between strains in terms of biofilm production or cold stress adaption (Di Bonaventura et al., 2008; Nufer, Stephan, & Tasara, 2007).

The finding of corresponding genotypes in the processing environment, pre-handled ingredients (e.g. salmon sliced in the plant), and sandwiches in the present study shows that the processing environment might contribute to the contamination of products, as it has been demonstrated before for other foods, especially for seafood (Lin et al., 2006; Martinez et al., 2003; Norton et al., 2001; Wulff et al., 2006).

The repeated finding of corresponding genotypes on certain localizations in the examined plant allowed the identification of plant-specific problem areas. In the first sampling phase (nine months), matching genotypes were repeatedly found on various localizations, but in particular on slicers and conveyor belts. On this equipment, corresponding genotypes were detected on repeated occasions and both during the working process and after cleaning and disinfection. Due to their construction, slicers or conveyor belts are often difficult to clean and maintain adequately and therefore constitute probable contamination sources for food products (Lin et al., 2006; Sheen & Hwang, 2008; Tolvanen, Lundén, Hörman, & Korkeala, 2009; Vorst, Todd, & Rysert, 2006). Moreover, air blow-guns and water hoses testing repeatedly positive for strains of certain genotypes might indirectly contributed to the contamination of food products by the hands of employees (Kerr, Birkenhead, Seale, Major, & Hawkey, 1993; Martinez et al., 2003; Montville, Chen, & Schaffner, 2001). Based on the data from the first sampling phase, cleaning and disinfection procedures of the plant were revised with a main focus on identified problem areas, including enhanced supervision by the quality assurance management. The second sampling phase (two months) evaluated the effect of the revised cleaning and disinfection schemes, and also included the examination of additional difficult to clean localizations of the processing environment. In this second phase, *Listeria* were no longer found in samples from slicers, conveyor belts, or products. However, *L. monocytogenes* were still detected sporadically in environmental samples (e.g. on water hoses), and the intensified examination identified the inside of a bread-feeding machine as further problem area. The four *L. monocytogenes* strains from the bread-feeding machine all belonged to the predominant genotype (Rep PCR profile a, PFGE profile A) and were obtained during the first two visits. After revision of the cleaning and disinfection scheme of this machine, *Listeria* were no longer found. Hence, the implementation of the revised cleaning and disinfection procedures effectively improved the *Listeria* situation in this ready-to-eat food processing plant.

An efficient food safety and hygiene concept of a food business operator must involve basic hygiene measures (good hygiene and manufacturing practices, including adequate cleaning and disinfection procedures), as well as product- and production-specific preventive measures in accordance with the HACCP principles (Sperber, 2005; Untermann, 1999; WHO, 1998). The results of the present study emphasize the importance of adequate cleaning and disinfection procedures in order to avoid *L. monocytogenes* becoming established in processing environments and thus posing a threat of product contamination. Furthermore, continuous environmental monitoring schemes for *L. monocytogenes* are of major importance to identify potential contamination sources and as an early warning system for food business operators, especially in food-processing plants with low *L. monocytogenes* prevalence in their food products.

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7. Tables

Table 1

Distribution of *Listeria* positive samples from the processing environment, ingredients, and final products of a sandwich-producing plant in the first sampling phase (November 2008 to July 2009).

	No. of samples	No. (%) of *L. monocytogenes* positive samples	No. (%) of *Listeria* spp. positive samples
Equipment of processing lines			
During working process	1192	46 (3.9)	1 (0.1)
After cleaning and disinfection	219	2 (0.9)	ND[a]
Environment			
During working process	307	13 (4.2)	ND
After cleaning and disinfection	61	3 (4.9)	ND
Ingredients[b] and final products	188	16 (8.5)	6 (3.2)

[a] ND, not detected.

[b] Ingredients pre-handled in the examined plant.

Table 2

Characterization of *L. monocytogenes* strains isolated from the processing environment.

	Origin	No. of strains	Sampling phase[a]	Sero-type	Genetic lineage	Rep PCR profile	PFGE profile
Equipment							
During working process	Slicers	27	1	1/2a	II	a	A
	Conveyor belts	11	1	1/2a	II	a	A
	Tables	5	1	1/2a	II	a	A
	Spattles	2	1	1/2a	II	a	A
	Gloves	1	1	1/2a	II	a	A
After cleaning and disinfection	Bread feeding machine	4	2	1/2a	II	a	A
	Conveyor belt	1	1	1/2a	II	a	A
	Slicer	1	1	1/2a	II	a	A
Environment							
During working process	Water hoses	3	1	1/2b	I	b	B
	Air blow-guns	2	1	1/2a	II	a	A
	Floors	2	1	1/2a	II	c	C
	Floor	1	1	1/2a	II	a	A
	Glove dispenser	1	1	1/2a	II	a	A
	Squeegee	1	1	1/2a	II	a	A
	Drain	1	1	1/2a	II	c	C
	Squeegee	1	1	1/2a	II	c	C
	Shelf	1	1	1/2a	II	c	C
After cleaning and disinfection	Water hose	1	1	1/2a	II	a	A
	Air blow-gun	1	1	1/2a	II	c	C
	Water hose	1	1	1/2a	II	c	C
	Drain	1	2	1/2a	II	c	C
	Water hose	1	2	1/2a	II	c	C

[a] 1, first sampling phase (November 2008 to July 2009); 2, second sampling phase (October to November 2009).

Table 3

Characterization of *L. monocytogenes* strains isolated from ingredients and final products.

Origin	No. of strains	Sampling phase[a]	Serotype	Genetic lineage	Rep PCR profile	PFGE profile
Sliced salmon[b]	4	1	1/2a	II	a	A
Tuna sandwiches	3	1	1/2a	II	c	C
Egg sandwiches	3	1	1/2a	II	a	A
Sliced salmon[b]	2	1	1/2b	I	f	F
Sliced salmon[b]	1	1	1/2a	II	d	D
Meat sandwich	1	1	1/2a	II	a	A
Meat sandwich	1	1	1/2b	I	e	E
Sliced ham[b]	1	1	1/2a	II	a	A

[a] 1, first sampling phase (November 2008 to July 2009).

[b] Sliced in the examined plant.

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